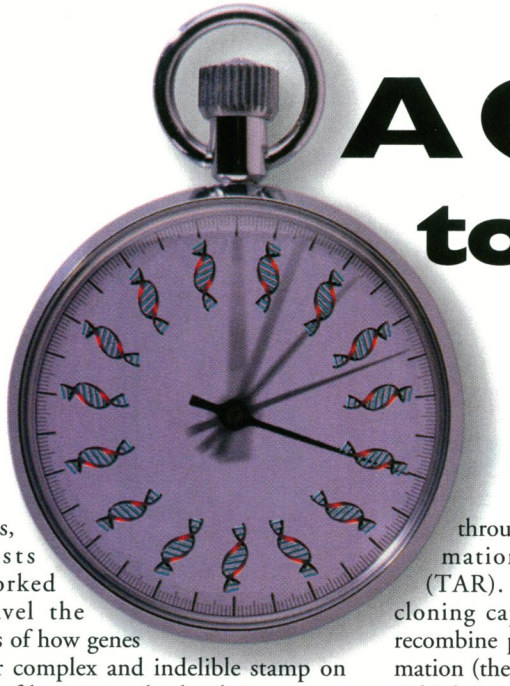


A Quicker Way to Clone

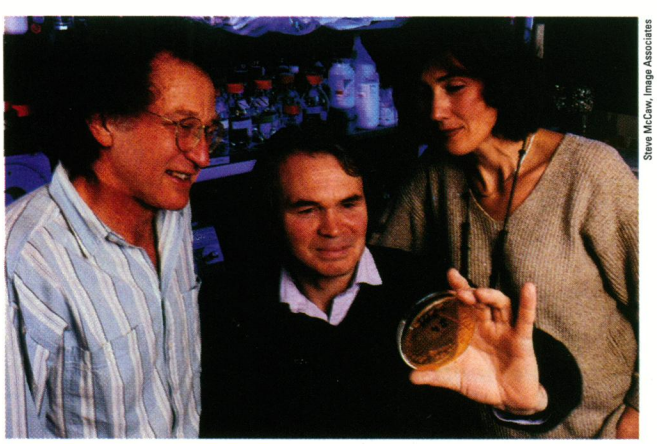
For years, scientists have worked to unravel the mysteries of how genes put their complex and indelible stamp on the lives of humans and other living organisms. In laboratories across the country, scientists scramble to identify the missing links in the chain of more than 100,000 estimated human genes. While the world awaits further advances that will bring the human genome into sharper focus, a group of scientists at the NIEHS has made an important genetic advance—an entirely new way to clone genes and parts of chromosomes—that may put the scientific community a few notches closer to understanding the role genes play in human disease.

Michael Resnick, a geneticist in the NIEHS Laboratory of Molecular Genetics, in collaboration with visiting Russian scientists Vladimir Larionov and Natalya Kouprina, has developed a new technique for swiftly isolating and cloning specific fragments of human genetic material directly into yeast cells. The resulting yeast artificial chromosomes (YACs) are engineered through a process known as transformation-associated recombination (TAR). According to Resnick, TAR cloning captures the ability of yeast to recombine pieces of DNA during transformation (the time during which DNA material is being introduced to a cell).

"This method will help us to focus on specific regions of human chromosomes in order to better understand origins of disease and to develop diagnosis of genetic diseases," says Francis S. Collins, director of the National Center for Human Genome Research, of the findings, which were published in the 9 January 1996 issue of the *Proceedings of the National Academy of Science.*

A Budding Collaboration

A mutual concern over the stability of human DNA in yeast played a part in bringing Resnick, Larionov, and Kouprina together to pursue this research. Larionov and Kouprina (both currently on leave from the Institute of Cytology in St. Petersburg, Russia) share Resnick's interest in this area, as the three discovered in 1988 when they met at a scientific meeting in Finland. Larionov, who heads the yeast genetics lab at the Institute of Cytology, has played a prominent role in yeast molecular biology in Russia, according to Resnick. A year later, Resnick invited Larionov to visit the NIEHS. They exchanged subsequent visits and a long-term transcontinental collaboration was born.



Steve McCaw, Image Associates

Yeast team. (left to right) Michael Resnick, Vladimir Larionov, and Natalya Kouprina examine some of their favorite subjects.

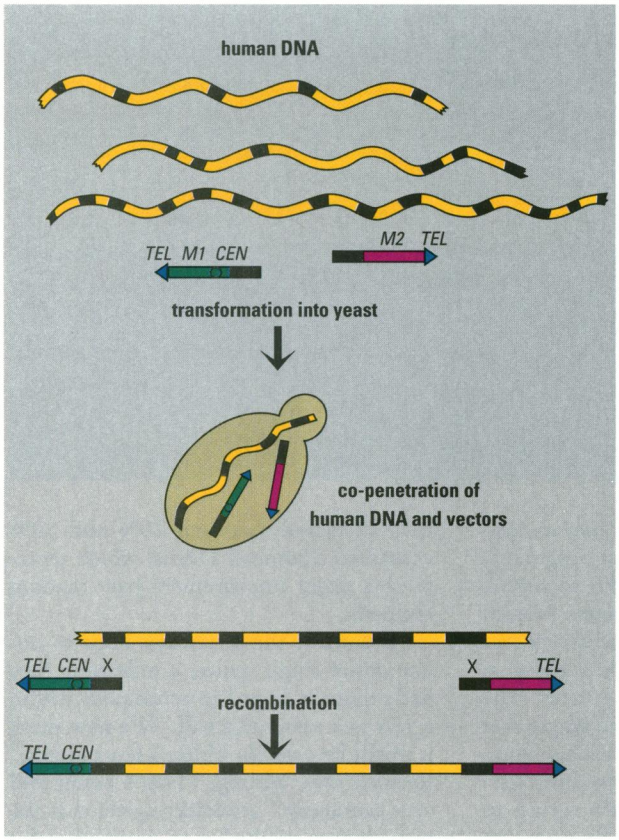
By the time the Russian-American team began its collaborative effort, human DNA's instability in yeast had begun to emerge as a serious problem in international human genetic research efforts. NIEHS researchers applied for and received funding from the Department of Energy and the National Center for Human Genome Research to study the underlying problem.

The traditional task of isolating and cloning DNA material involves randomly cutting DNA from cells into thousands and even millions of individual pieces. Each piece has to be treated so that it can be cloned into yeast or bacteria. Once cloning is complete, each fragment must be analyzed to find the desired fragment or gene. It's a random, hit-or-miss process defined mostly by endless hours of hair-splitting tedium.

With TAR cloning, however, scientists can rely on the yeast to find the precise sequence of interest. For this reason, Resnick refers to the new technique as a "smart" system. "Yeast has the ability to find the same or related molecules," explains Resnick, "and when it finds them, it also has the ability to join them to others." This uncanny ability to find and combine similar DNAs is independent of whether the DNAs originate in humans, animals, or plants. Human DNAs are introduced, or transformed, into yeast cells accompanied by a tiny piece of the gene or fragment that is ultimately desired. Only DNA that contains a region that matches that tiny piece is maintained, or cloned, as the yeast cells reproduce.

TAR Cloning

The team has been dealing primarily with large molecules, and is now exploring the maximum size that can be cloned. Currently the average size clone is approximately 250–300 kilobases (kb). Approximately 30% of human DNA contains short interspersed repeated sequences called SINEs. A specific subclass of SINEs called Alu sequences is used in TAR



Cloning chromosomes. Human DNA is isolated and transformed into yeast along with genetic markers (M1 and M2). YACs are created by recombination between Alu sequences (black blocks). Source: Larionov V et. al. Specific cloning of human DNA as yeast artificial chromosomes by transformation-associated recombination. *Proc Natl Acad Sci USA 93:1491–496 (1996).*

cloning. Alu sequences measure 300 bases in length; in the human genome, the average distance between Alu sequences is about 2–3 kb.

In the TAR cloning process, tiny plasmids of DNA, known as vectors, are transformed along with human DNA into yeast spheroplasts. At the end of each vector is a piece of DNA that corresponds to a repeat present on the human DNA and a genetic marker. According to Resnick, the end piece can be either precisely equal or simply related (i.e., diverged) by 10–20%. Once inside the cell, these vectors find their corresponding partner—either their relative or a precise match—on a chromosomal DNA fragment. At this point, a repeat in the chromosomal DNA fragment sequence joins by covalent linkage with a repeat on the vector through recombination, generating an artificial chromosome similar to the yeast cell's own chromosome.

Summing up one of the TAR method's greatest advantages, Marvin Stodolsky says, "It's quick and fast and there's no risky test-tube preparation of recombinant DNAs." Stodolsky is an administrator with the Department of Energy's Human Genome Task Group, which initially provided support for Resnick's team's work. "It provides a nice way to reclone economically," he adds, "and is thus a nice addition to the tableaux of genetic research done today."

Faster, Cleaner Cloning

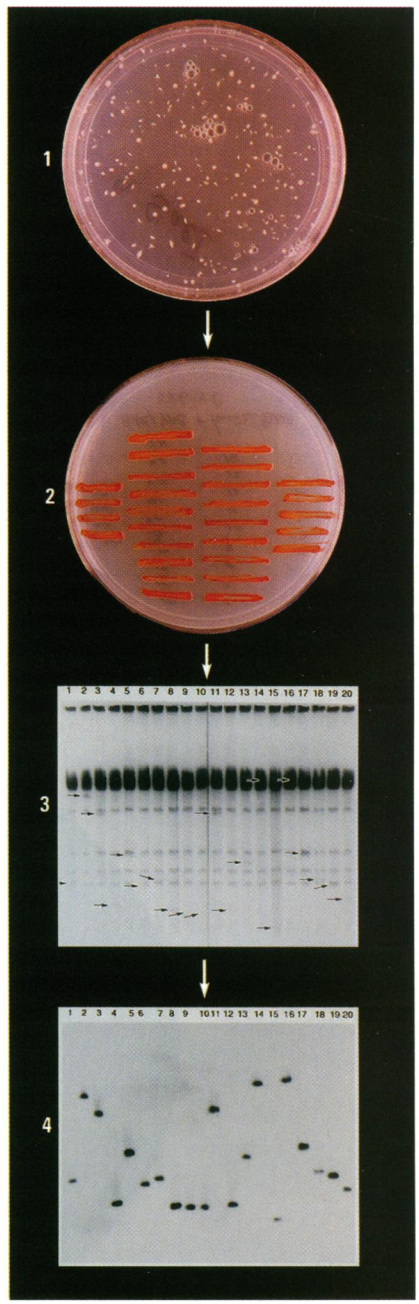
Maynard V. Olson, a professor of molecular biotechnology at the University of Washington in Seattle who has worked extensively in genome analysis, also praises the NIEHS findings. "It was unusually interesting," says Olson. "I think the results are somewhat surprising. They point the way toward a tremendous simplification in developing a clone library."

The original method for cloning genes was developed in Olson's laboratory 10 years ago. Since then, at least a thousand scientific papers have been written making use of these clones, with only three or four libraries carrying the weight of all this work, Olson says. Until now, libraries have been difficult to build, and Olson hopes this new technique will enable other scientists to develop their own libraries using their own material. "Though libraries have been widely used, it has stopped progressing," says Olson of the current cloning technique. "This is the first development in several years."

Besides saving time, the new cloning method sidesteps the land mines that typically litter the path toward traditional gene cloning. "One thing that's absolutely key is that the technique involves really a minimum handling of human DNA," says Resnick. Handling—or, more accurately, mishandling—may account for some of the chimeras (hybrid DNA molecules that arise by interactions between two or more noncontiguous DNA fragments) usually associated with cloning. With the old technique, the tricky process of managing DNA material includes extracting and isolating the DNA as gently as possible, restricting the DNA, and joining it to vector DNA, often followed by a reisolation—all before introducing the DNA into the yeast for cloning. "We think all those steps were sources of problems that could lead to instability," Resnick explains. "We know that if the DNA has lots of nicks in it, that could stimulate instability when the DNA goes into yeast."

Exploring the Gene–Disease Link

So far, the NIEHS team has isolated DNA regions from chromosomes 10, 16, and 22—regions that harbor several known disease genes. TAR cloning opens the door to



Treasure hunt. Fragments of human DNA are isolated from YACs created by TAR cloning (1 and 2) and analyzed by probing chromosomal gels (3) with a human DNA-specific probe. Dark spots (4) indicate human DNA.

cloning specific genes or other chromosome segments that are related to disease, Resnick says. So far, the promoter regions of genes that have been isolated and identified have been poorly understood. "The promoter region of the gene is what controls its expression," explains Resnick. "We think this might provide opportunities to more rapidly get many copies of promoters. We also foresee that this would be very useful in characterizing the genes that play a role in responses to genotoxins—either making us more susceptible or enabling us to deal with damage better," Resnick predicts.

Larionov, Kouprina, and Resnick are now joining others at the NIEHS, including Scientific Director J. Carl Barrett, in a quest to apply this new isolation and cloning method to further research in genetic diseases. Barrett's laboratory has played a key role in identifying breast cancer and prostate cancer suppressor genes.

In another experiment, the team successfully used the Alu repeat to clone human DNA that had been introduced into mouse cells. "Mouse cells do not have Alus," Resnick explains, "so there's no way to recombine them." This feature makes human/mouse hybrid cells useful for characterizing and isolating human genes. Once they completed the experiment, the team found a 30- to 60-fold enrichment of human DNA in the mouse cells. The amount of human DNA originally present in the mouse cells was only 2% of the total DNA. According to Resnick, chimeras formed by cloning unrelated human fragments or human and mouse fragments into a YAC confounded the scientists working on the Human Genome Project. With the TAR method, however, no chimeras were recovered. "We're going to be refining this," Resnick assures. The team has now developed a technique that enables them to get rid of excess background DNA plasmids and false positives.

The NIEHS team is also trying to determine just how efficiently they can extract DNA. "We've just done a set of experiments trying to isolate a family of genes—the ribosomal DNA family—and instead of using Alu, we're using a segment of ribosomal DNA." Of the YACs they extracted, between 20% and 50% contained ribosomal DNAs, which represents a major improvement over random methods.

Eventually the researchers hope to isolate entire single genes, a milestone they had originally hoped to accomplish within a year to a year and a half. "We now think it might be possible within a few months," Resnick says, adding, "That's being said very cautiously." Stodolsky agrees that the new cloning method may prove ideal for just that: "If you want to look at gene-sized clones, then the NIEHS process becomes very effective."

Jennifer Medlin

Suggested Reading

Ketner G et al. Efficient manipulation of the human adenovirus genome as an infectious yeast artificial chromosome clone. Proc Nat Acad Sci USA 91:6186–6190 (1994).

Larionov V et al. Specific cloning of human DNA as YACs by transformation-associated recombination. Proc Nat Acad Sci USA 93:491–496 (1996).

Burke DT et al. Cloning of large segments of DNA into yeast by means of artificial chromosome vectors. Science 226:806–812 (1987).

Nelson DL Brownstein BH, eds. YAC Libraries: A user's guide. New York:W.H. Freeman and Co., 1993.

Shizuya H et al. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in Escherichia coli using an F-factor-based vector. Proc Nat Acad Sci USA 89: 8794–8797 (1992).